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(54) Title: **THE DELAY, PREVENTION AND/OR REVERSAL OF CELL SENESENCE**

(57) Abstract

The present invention provides a method for the delay, prevention and/or reversal of cell senescence. The method of the present invention comprises administering to the cells a composition including a compound having chemical properties similar to carnosine, the active compound being present in a concentration of greater than 10mM. In preferred forms of the invention the compound is carnosine or homocarnosine and the cells are preferably human fibroblast cells. The invention also provides an improved cell culture medium which includes carnosine or a carnosine like compound.



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THE DELAY, PREVENTION AND/OR REVERSAL OF CELL SENESCENCE
Field of the Invention

The present invention relates to a method for the delay, prevention and/or reversal of cell senescence. In particular the invention has applicability to the delay, prevention and/or reversal of senescence in fibroblast cells. The present invention further relates to an improved cell culture medium.

Background of the Invention

10 Carnosine is a dipeptide, β -alanyl-L-histidine, which is present in high amounts in tissues such as brain and muscle. Its function is unknown, but there are reports that it may act as an antioxidant and help protect cells from free radical damage. It has been shown that
15 carnosine prevents collagen and protein crosslinking and this discovery was the basis for International Patent Application No W090/06102 entitled "Compound and Method for the Retardation of Collagen Cross-Linking". Such cross-linking is well known to be associated with aging.

20 Human diploid fibroblasts constitute an important part of the skin. They secrete collagen, which is a structural component, and are active in wound healing. Small skin biopsies in normal culture medium yield primary cultures of dividing fibroblasts. These cells can be
25 subcultured, or passaged, many times and they retain for a considerable period their characteristic morphology and diploid chromosome number.

The cells, however, eventually grow more slowly and an increasing proportion stop dividing. This is
30 accompanied by significant morphological changes. Whereas young healthy fibroblasts are of uniform size and line up in parallel arrays to form characteristic "whorls" of cells in a confluent monolayer, cells which have divided many times in culture become more irregular in size, with
35 many large cells, more granular in appearance, and they no

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longer form regular whorls. Such cells may grow slowly for several more divisions, but eventually cell division ceases and the population fails to form a confluent monolayer. At this time the cells are extremely
5 heterogeneous in appearance, and many eventually detach from the substrate.

The limited life span of cultured human fibroblasts is sometimes referred to as the "Hayflick limit", after the discoverer of this phenomenon. Hayflick proposed that
10 the cessation of growth was due to an intrinsic process which lead to terminal senescence, and that cultured human fibroblasts provide a suitable experimental model for the study of cellular ageing. His view has been confirmed by the finding that skin cells derived from biopsies from old
15 individuals have a significantly shorter life span than those from young individuals. Also, in comparative studies with different mammalian species, it has been shown that the in vitro life span of their fibroblasts is directly related to the normal life span of each donor
20 animal. Although innumerable investigations have documented in detail the limited in vitro life span of human fibroblasts and also other cell types, and many important experimental studies have been carried out, there is no agreement about the likely molecular basis of
25 senescence and ageing in these cells.

It is known that carnosine aids growth and stimulates collagen synthesis in embryonic chick fibroblasts in culture (Han et al, 1985) and aids wound healing (Nagai et al, 1986). There are no prior claims however that
30 carnosine affects ageing and senescence in tissue culture in the way described in this document. (references:- Han et al (1985) Korean Biochem. J. 18, 417-422; Nagai et al (1986) Surgery 100, 815-21).

The present inventors have discovered that the
35 senescence of cells, particularly human fibroblast cells,

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may be delayed, prevented and/or reversed by maintaining the cells in the presence of an effective amount of a compound having chemical properties similar to carnosine. The chemical properties of carnosine include its ability
5 to scavenge oxidative free radicals and its capacity to chelate certain cations including copper. Compounds having chemical properties similar to carnosine include carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine, carcinine, a compound of the
10 formula R_1 -L(or D)-His- R_2 , cosmetically or pharmaceutically acceptable salts thereof and combinations thereof, in which R_1 = L-(or D)-lysine, or a homologue, optionally α -amino acylated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, and
15 R_2 = L-(or D)-lysine, or a homologue, optionally α -carboxyl esterified or amidated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms.

It has been well established by experiments performed
20 by a number of independent groups that there is a close relationship between the passaging ability of human primary tissue culture cells and the tissue age of the donor. So, whilst a fibroblast culture established from tissues of a new born baby can be passaged through some
25 55 - 60 cell divisions before reaching its "Hayflick limit" and before dying, a similar culture established from a 60 year old can be passaged through only 10 or fewer cell divisions before dying. A culture established from a 30 year old has a "Hayflick limit" intermediate in
30 size of between 10 and 60 cell divisions. The Hayflick limit of cells taken from a donor can be considered an index of the state of senescence of the tissues of the donor.

Ageing of human primary cell cultures are considered
35 an accurate model of ageing of the whole human being.

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Factors which affect ageing of cells in culture are believed of importance in ageing of tissues and the whole man. Generally, such studies have been used only to identify factors of which there are many which diminish survival expectations. Factors which prolong survival or which rejuvenate cells are exceptionally rare. The inventors know of only one other report where prolongation of human cell survival is well substantiated and that was for a non-peptide compound.

It will be obvious to those skilled in the biomedical science of ageing that a substance which extends the life of human primary fibroblasts in culture and which can rejuvenate senescent cultures which are close to their "Hayflick Limit" will be likely to have a similar effect in tissues in the whole human person - if delivered to that tissue at an appropriate rate and in an appropriate form.

Consequently, it is believed that a compound having chemical properties similar to carnosine would be useful in slowing down ageing in normal and diseased patients and rejuvenating such patients who already are senescent.

Moreover, they will have a beneficial effect on age-related diseases or conditions, such as Alzheimers Disease and other age-related neurodegenerative conditions. In addition such a compound should have application in cosmetics to rejuvenate skin and to assist the skin in its ability to renew itself and to slow down the aging process.

Summary of the Invention

Accordingly, in a first aspect the present invention consists in a method of delaying, preventing and/or reversing senescence of cells comprising applying to the cells an effective amount of a composition, the composition including an active compound(s) having chemical properties similar to carnosine, the active

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compound being present in the composition at a concentration of at least 10mM.

In a second aspect the present invention consists in a method of treating an age-related disease in a subject
5 suffering from such a disease comprising administering to the subject an effective amount of a composition, the composition including an active compound(s) having chemical properties similar to carnosine, the compound being present in the composition at a concentration of at
10 least 10mM.

In a third aspect the present invention consists in a method of rejuvenating skin, assisting the skin in its ability to renew itself and/or reducing aging of skin of a subject the method comprising administering to the subject
15 an effective amount of a composition, the composition including an active compound(s) having chemical properties similar to carnosine, the active compound being present in the composition at a concentration of at least 10mM.

In a preferred embodiment of the present invention
20 the active compound(s) is selected from the group consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine, carcinine, a compound of the formula R_1 -L(or D)-His- R_2 , cosmetically or pharmaceutically acceptable salts thereof
25 and combinations thereof, in which R_1 = L-(or D)-lysine, or a homologue, optionally α -amino acylated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, and R_2 = L-(or D)-lysine, or a homologue, optionally α -carboxyl esterified or amidated with
30 alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms. The histidine residue of the active compound(s) may be modified such that it is of the formula R_3 -L(or D)-His(R_4)- R_5 , where R_3 = w-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms;
35 R_4 = void, or imidazole modification with alkyl,

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5 sulphhydryl, hydroxyl, halogen, and/or amino groups;
R₅ = carboxyl (alkyl) amides with 1 to 12 carbon atoms,
preferably 2 to 6 carbon atoms. The active compound(s)
may also include isosteric amide bonds and/or isosteric
peptide bonds.

Examples of such modified compounds include
AcLys-HisNH₂, AcHis-LysNH₂ and AcLys-His-LysNH₂
oligomeric forms of the active compounds may also be used
such as (β-Ala-His)_n and (Lys-His)_n where n = 2-5.

10 In a preferred embodiment of the present invention
the active compound is carnosine or homocarnosine and is
most preferably carnosine.

While it is presently preferred that the active
compound is carnosine or homocarnosine a number of
15 synthetic compounds referred to above may have advantages
over the natural compounds in terms of efficacy,
deliverability, toxicity, economy of production and
stability both chemical and metabolic. It is preferred
that the active compound is present in the composition in
20 a concentration range of 10mM to 100mM, more preferably
10mM to 50mM and most preferably 15mM to 30mM.

In a further preferred embodiment of the present
invention the cells are human fibroblast cells.

The composition may be administered to the cells in a
25 culture medium in the case of cells being cultured
in vitro. In the case of in vivo cells the composition
may be applied in any suitable manner such as injection,
infusion, ingestion, inhalation, iontophoresis,
electroporation, or topical application.

30 It is presently preferred, however, that the
composition is administered topically.

In a preferred embodiment the active compound(s) is
mixed with or linked to another molecule(s), which
molecule is such that the composition is improved in
35 regard to skin penetration, skin application, tissue

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absorption/adsorption, skin sensitisation and/or skin irritation.

The molecule(s) is preferably selected from the group consisting of sodium lauryl sulphate, lauryl amonium
5 oxide, ozone, decylmethyl sulphoxide, lauryl ethoxylate, octanol, dimethyl sulphoxide, propylene glycol, nitroglycerine, ethanol and combinations thereof.

The active compound may also be part of delivery system comprising fatty acids, lipids, liposomes, amino
10 acids, peptides, polypeptides, proteins, carbohydrates, alcohols, polyalcohols, vitamins, anti-oxidants, preservatives, fragrances, organic or water based solvents and mixtures thereof.

In a further preferred embodiment of the present
15 invention the composition includes a compound selected from the group consisting of bilirubin, carotenoids, mannitol, sorbitol, glutathione, selenium, uric acid, vitamin A, vitamin C, vitamin E and combinations thereof. It is also preferred that the pH of the composition is
20 adjusted to optimise activity of the active compound and/or to improve tissue uptake.

In yet another preferred embodiment of the present invention the active compound is mixed with or linked to another molecule, which molecule is such that the
25 composition is improved in regard to blood brain barrier penetration.

In a fourth aspect the present invention consists in an improved culture medium, the improvement comprising including in the cell culture medium an active compound
30 having chemical properties similar to carnosine at a concentration greater than 10mM.

The invention will hereinafter be described with reference to carnosine for convenience, however, this is not to be taken as a limitation on the broad scope of the
35 present invention.

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Detailed Description of the Invention

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples

5 and figures in which:-

Figure 1 shows confluent young MRC-5 cells;

Figure 2 shows senescent MRC-5 cells;

Figure 3 shows RR cells grown continuously in 30mM carnosine;

10 Figure 4 shows RR cells growing continuously in 50mM carnosine;

Figure 5 shows growth of MRC-5 cells from early passage to senescence and no subsequent growth in normal medium (DMEM) and medium supplemented throughout with 20mM (0-0) and 30mM (x-x) carnosine. Control cells were also transferred to these concentrations of carnosine at the time indicated by the arrows (see Figure 8);

Figure 6 shows untreated senescent MRC-5 cells at PD level 61 which have reached the end of their lifespan;

20 Figure 7 shows cells growing continuously in 20mM carnosine at PD level 67;

Figure 8 shows the growth of MRC-5 cells after transfer from normal medium to medium containing 20mM (0-0) or 30mM (x-x) carnosine at PD level 55. The control cells (0-0) ceased growth at PD level 61 (dashed line);

25 Figure 9 shows MRC-5 cells grown in medium containing 30mM carnosine after transfer from normal medium at PD level 55. The ordinant indicates the yield of cells per flask at each sub-culture. The increase in cell yield corresponds to the increased growth rate shown in Figure 8;

30 Figure 10 shows control MRC-5 cells at 55 PDs beginning to show clear signs of senescence. These cells were transferred to 20mM and 30mM carnosine;

Figure 11 shows the control cells growing in 20mM carnosine 146 days after transfer at 64 PDs; and

35

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Figure 12 shows control cells growing in 30mM carnosine 146 days after transfer at 63 PDs (photographed at lower magnification to show the whorls characteristic of young human fibroblasts).

5 Effect of Carnosine on Human Fibroblast Growth and Lifespan
RR Cells

Cells were grown in Dulbecco's modification of Eagles' Minimal Essential Medium, with 10% fetal calf serum. When cells became confluent and ceased division, they were
10 harvested with trypsin - versene, and dispersed to form a suspension of single cells which was counted with a Coulter Counter. Depending on the cell yield the culture was split in a ratio of 1:2, 1:4, or 1:8. These split ratios correspond approximately to one, two or three cell
15 population doublings when the new cultures become confluent. The exact number of population doublings is calculated from the cell yield at each sub-culture.

Initially, human foreskin fibroblasts were obtained from Dr Roger Reddel (Children's Medical Research
20 Foundation) at passage 5, flasks were set up with differing concentrations of carnosine (10mM, 20mM, 30mM and 50mM) together with control cultures. Each of the cultures were passaged in the same medium.

These RR cells grew as rapidly as control cultures
25 when 10mM and 20mM carnosine was added to standard medium. With 30mM there was some reduction in growth rate, and with 50mM a great reduction. After many population doublings, the cells grown in 30mM and 50mM retained their characteristic fibroblast morphology, that
30 is, the cells are elongated, line up in parallel arrays to form characteristic whorls of growth, and exhibit contact inhibition of cell division.

It is significant that the cultures in high levels of carnosine (30mM and 50mM) retained their fibroblast
35 morphology, and are still growing after 500 days

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(Figures 3 and 4)(for comparison see Figures 1 and 2).

MRC-5 cells

MRC-5 is a very well characterised fibroblast strain derived from foetal lung tissue. Early passage cultures were obtained from the National Institute for Biological Standards and Control, South Mimms, Herts, U.K. Longevity experiments were set up using normal medium and medium supplemented with 20 and 30mM carnosine (medium was the same as for RR cells above). There was little difference in growth rate in the initial stage. The control cells became senescent at the expected time, namely after about 50 - 55 PDs, and they ceased growth at 57 and 61 PDs. The cells in 20mM and 30mM carnosine had very significantly increased longevity (Table 1 and Figure 5). It should be noted that the PD level is a logarithmic measure of growth, so an increase of 10 PDs represents an increase in cell mass of 2^{10} , or x 1,000. Carnosine also delayed the normal morphological features of senescence (Figure 7 in comparison to Figure 6). When control cells become senescent, the yield of cells per flask declines, but carnosine significantly increases the number of cells per flask as the end of the culture lifespan approaches.

Untreated cells which had slowed down and exhibited a senescent phenotype were transferred at PD level 55 to 20mM and 30mM carnosine. These cultures continued to grow slowly, but with a rejuvenated phenotype. Remarkably, these cells are continuing to grow after 400 days (Figure 8), whereas cells grown continuously in carnosine died out after 139 days. Thus it appears that transfer of senescent cells to carnosine can reverse the senescent phenotype and allow extended growth of cells with a near normal morphology (Figures 10, 11 and 12). The effect of carnosine in increasing cell yield was particularly striking in cells transferred from normal medium to 30mM carnosine medium (Figure 9).

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TABLE 1

Longevity experiments with human fibroblasts, strain MCR-5. Cells were grown in medium with or without carnosine, as indicated.

CARNOSINE TREATMENT	NUMBER OF CULTURES	LIFESPAN IN POPULATION DOUBLINGS	DAYS IN CULTURE*	
Control - none	2	56.7, 60.6	98, 139)
20mM	2	70.7, 69.4	139, 139)
30mM	2	64.7, 63.5	139, 139)
Transfer from control:				
PD 55.3 - 20mM	1	69.0	378*)
PD 55.1 - 30mM	1	68.6	378*)

* "Days in culture" refers to the date of the last split (subculture). The cultures transferred from normal medium to carnosine are still viable at 401 days.

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As will be clear from the above results the present inventors have shown that senescence of cells may be delayed, prevented and/or reversed by the administration of carnosine.

5 In addition an improved cell culture medium is provided which includes carnosine at a concentration of greater than 10mM. This culture medium can be of any of the large range of culture media known to persons skilled in this field to which carnosine has been added.

10 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments
15 are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:-

1. A method of delaying, preventing and/or reversing senescence of cells comprising applying to the cells an effective amount of a composition, the composition
5 including an active compound(s) having chemical properties similar to carnosine, the active compound being present in the composition at a concentration of at least 10mM.
2. A method of treating an age-related disease in a subject suffering from such a disease comprising
10 administering to the subject an effective amount of a composition, the composition including an active compound(s) having chemical properties similar to carnosine, the compound being present in the composition at a concentration of at least 10mM.
- 15 3. A method of rejuvenating skin, assisting the skin in its ability to renew itself and/or reducing aging of skin of a subject the method comprising administering to the subject an effective amount of a composition, the composition including an active compound(s) having
20 chemical properties similar to carnosine, the active compound being present in the composition at a concentration of at least 10mM.
4. A method as claimed in any one of claims 1 to 3 in which the active compound(s) is selected from the group
25 consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine, carcinine, a compound of the formula R_1 -L(or D)-His- R_2 , cosmetically or pharmaceutically acceptable salts thereof and combinations thereof, in which R_1 = L-(or D)-lysine,
30 or a homologue, optionally α -amino acylated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, and R_2 = L-(or D)-lysine, or a homologue, optionally α -carboxyl esterified or amidated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2
35 to 6 carbon atoms.

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5. A method as claimed in claim 4 in which the histidine residue of the active compound(s) is of the formula R_3 -L(or D)-His(R_4)- R_5 , where R_3 = w-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms;
- 5 R_4 = void, or imidazole modification with alkyl, sulphhydryl, hydroxyl, halogen, and/or amino groups; R_5 = carboxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms.
6. A method as claimed in any one of claims 1 to 5 in
- 10 which the active compound(s) includes isosteric amide bonds and/or isosteric peptide bonds.
7. A method as claimed in any one of claims 1 to 4 in which the active compound is carnosine or homocarnosine.
8. A method as claimed in claim 7 in which the active
- 15 compound is carnosine.
9. A method as claimed in any one of claims 1 to 6 in which the active compound(s) is (β -Ala-His) $_n$ or (Lys-His) $_n$ wherein $n = 2-5$.
10. A method as claimed in any one of claims 1 to 9 in
- 20 which the active compound(s) is present in a concentration of between 10mM and 100mM.
11. A method as claimed in claim 10 in which the active compound is present in a concentration of between 10mM and 50mM.
- 25 12. A method as claimed in claim 11 in which the active compound is present at a concentration of between 15mM and 30mM.
13. A method as claimed in any one of claims 1 or 4 to 10 in which the cells are human fibroblast cells.
- 30 14. A method as claimed in any one of claims 1 to 11 in which the composition is administered by injection, infusion, ingestion, inhalation, iontophoresis, electroporation, or topical application.
15. A method as claimed in claim 14 in which the
- 35 composition is applied topically.

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16. A method as claimed in any one of claims 1 to 15 in which the active compound(s) is mixed with or linked to another molecule(s), which molecule is such that the composition is improved in regard to skin penetration,
5 skin application, tissue absorption/adsorption, skin sensitisation and/or skin irritation.
17. A method as claimed in claim 16 in which the molecule(s) is selected from the group consisting of sodium lauryl sulphate, lauryl ammonium oxide, ozone,
10 decylmethyl sulphoxide, lauryl ethoxylate, octanol, dimethyl sulphoxide, propylene glycol, nitroglycerine, ethanol and combinations thereof.
18. A method as claimed in any one of claims 1 to 17 in which the active compound is part of delivery system
15 comprising fatty acids, lipids, liposomes, amino acids, peptides, polypeptides, proteins, carbohydrates, alcohols, polyalcohols, vitamins, anti-oxidants, preservatives, fragrances, organic or water based solvents and mixtures thereof.
- 20 19. A method as claimed in any one of claims 1 to 18 in which the composition includes a compound selected from the group consisting of bilirubin, carotenoids, mannitol, sorbitol, glutathione, selenium, uric acid, vitamin A, vitamin C, vitamin E and combinations thereof.
- 25 20. A method as claimed in any one of claims 1 to 19 in which the pH of the composition is adjusted to optimise activity of the active compound and/or improve tissue uptake.
21. A method as claimed in any one of claims 1, 2 or 4
30 to 10 in which the active compound is mixed or linked to another molecule, which molecule is such that the composition is improved in regard to blood brain barrier penetration.
22. An improved culture medium, the improvement
35 comprising including in the cell culture medium an active

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compound having chemical properties similar to carnosine at a concentration greater than 10mM.

23. An improved culture medium as claimed in claim 22 in which the active compound is selected from the group
- 5 consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine, carcinine, a compound of the formula R_1 -L(or D)-His- R_2 , pharmaceutically acceptable salts thereof and combinations thereof, in which R_1 = L-(or D)-lysine, or a homologue,
- 10 optionally α -amino acylated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, and R_2 = L-(or D)-lysine, or a homologue, optionally α -carboxyl esterified or amidated with alkyl or aralkyl with 1 to 12 carbon atoms, preferably 2 to 6
- 15 carbon atoms.

24. An improved culture medium as claimed in claim 23 in which the histidine residue of the active compound(s) is of the formula R_3 -L(or D)-His(R_4)- R_5 , where R_3 = w-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6
- 20 carbon atoms; R_4 = void, or imidazole modification with alkyl, sulphydryl, hydroxyl, halogen, and/or amino groups;

R_5 = carboxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms.

25. An improved culture medium as claimed in any one of claims 22 to 24 in which the active compound(s) includes isosteric amide bonds and/or isosteric peptide bonds.
26. An improved culture medium as claimed in either claim 22 or claim 23 in which the active compound is carnosine
- 30 or homocarnosine.
27. An improved culture medium as claimed in claim 26 in which the active compound is carnosine.
28. An improved culture medium as claimed in any one of claims 22 to 25 in which the active compound(s) is
- 35 $(\beta\text{-Ala-His})_n$ or $(\text{Lys-His})_n$ wherein $n = 2-5$.

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29. An improved culture medium as claimed in any one of claims 22 to 28 in which the active compound(s) is present in a concentration of between 10mM and 100mM.

30. An improved culture medium as claimed in claim 29 in
5 which the active compound is present in a concentration of between 10mM and 50mM.

31. An improved culture medium as claimed in claim 30 in which the active compound is present at a concentration of between 15mM and 30mM.

[illegible]

The first of these is the fact that the
 Government has been unable to secure the
 necessary funds to carry out its policy.
 The second is the fact that the Government
 has been unable to secure the necessary
 funds to carry out its policy.

1

FIGURE 1

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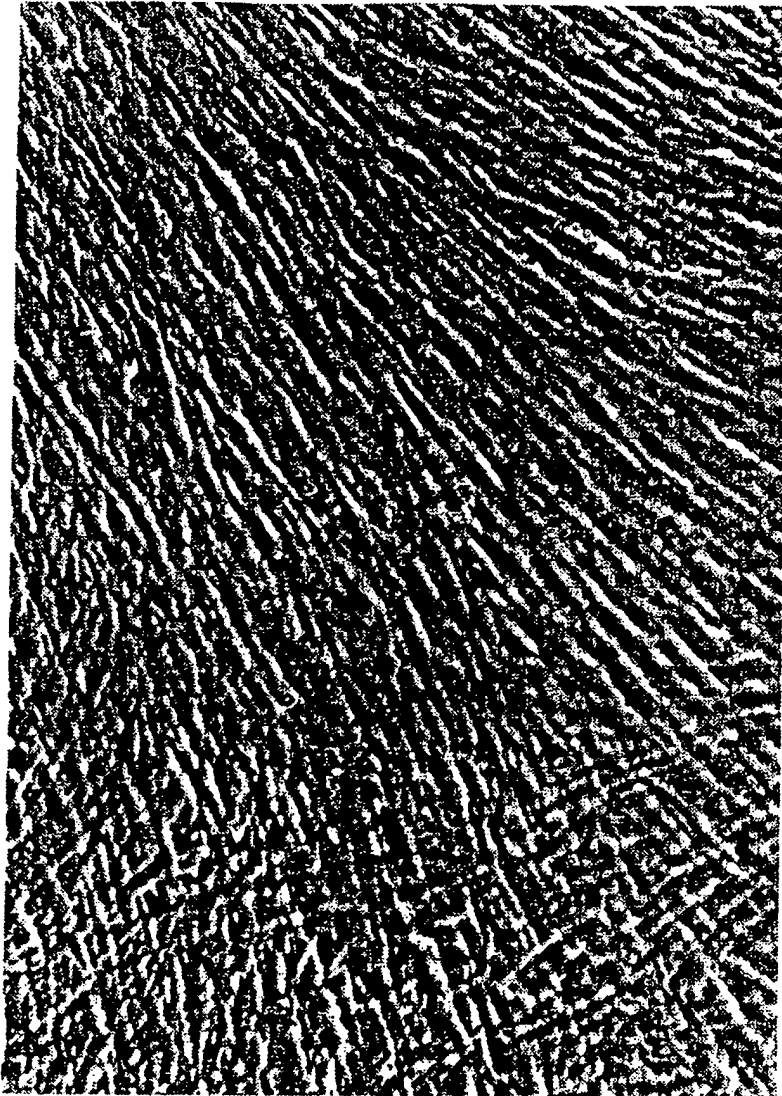


FIGURE 2

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FIGURE 3

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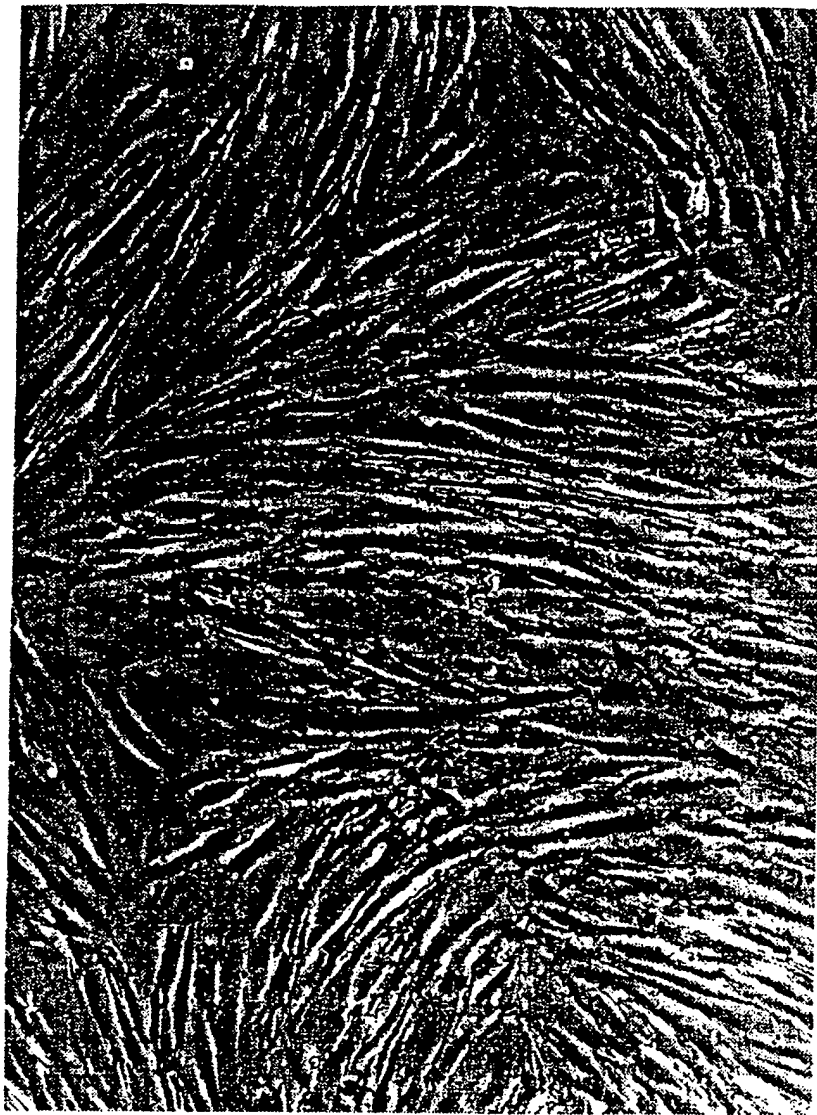


FIGURE 4

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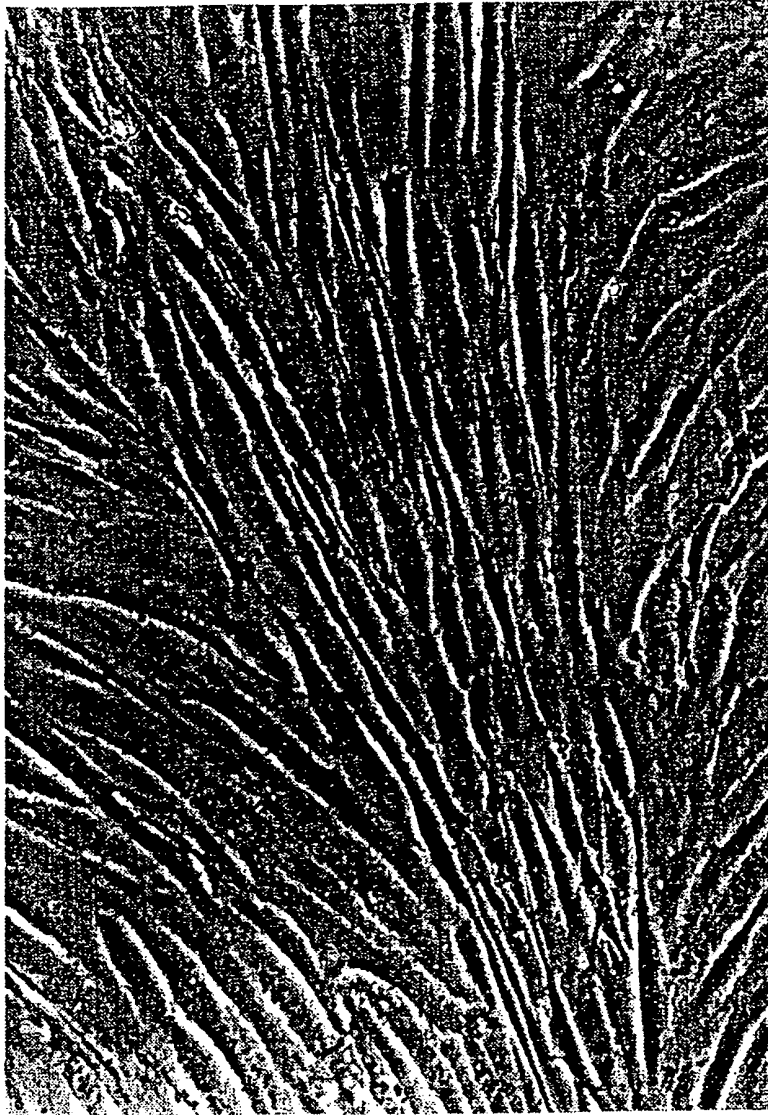


FIGURE 5

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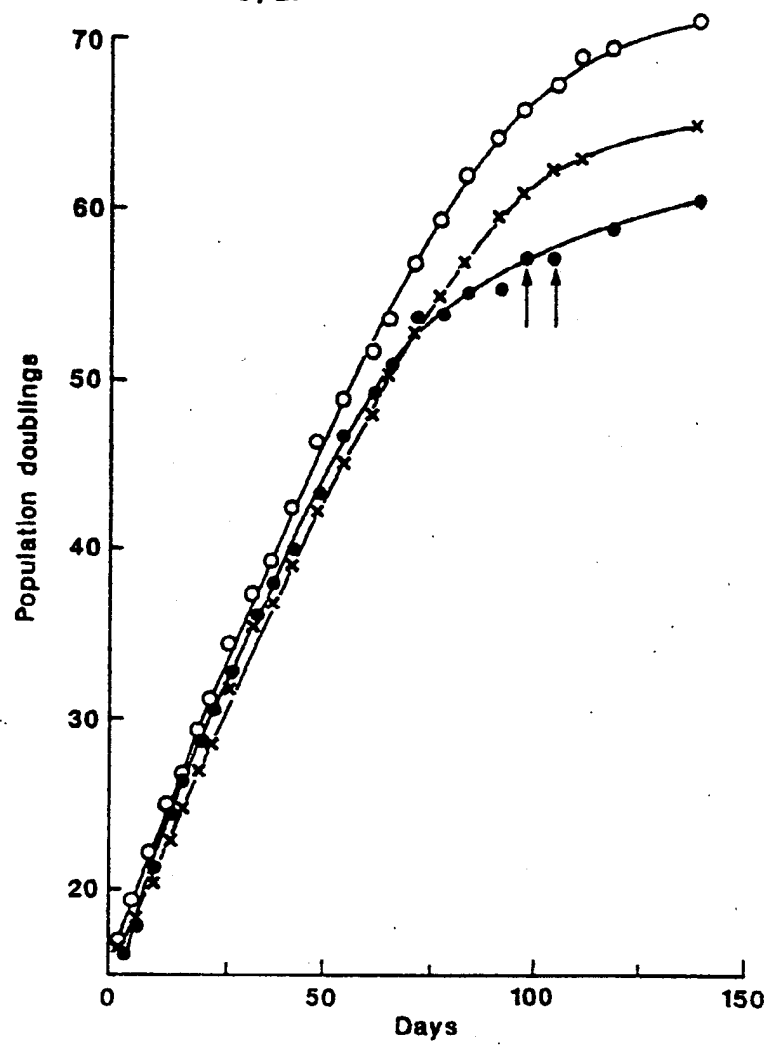


FIGURE 6

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FIGURE 7

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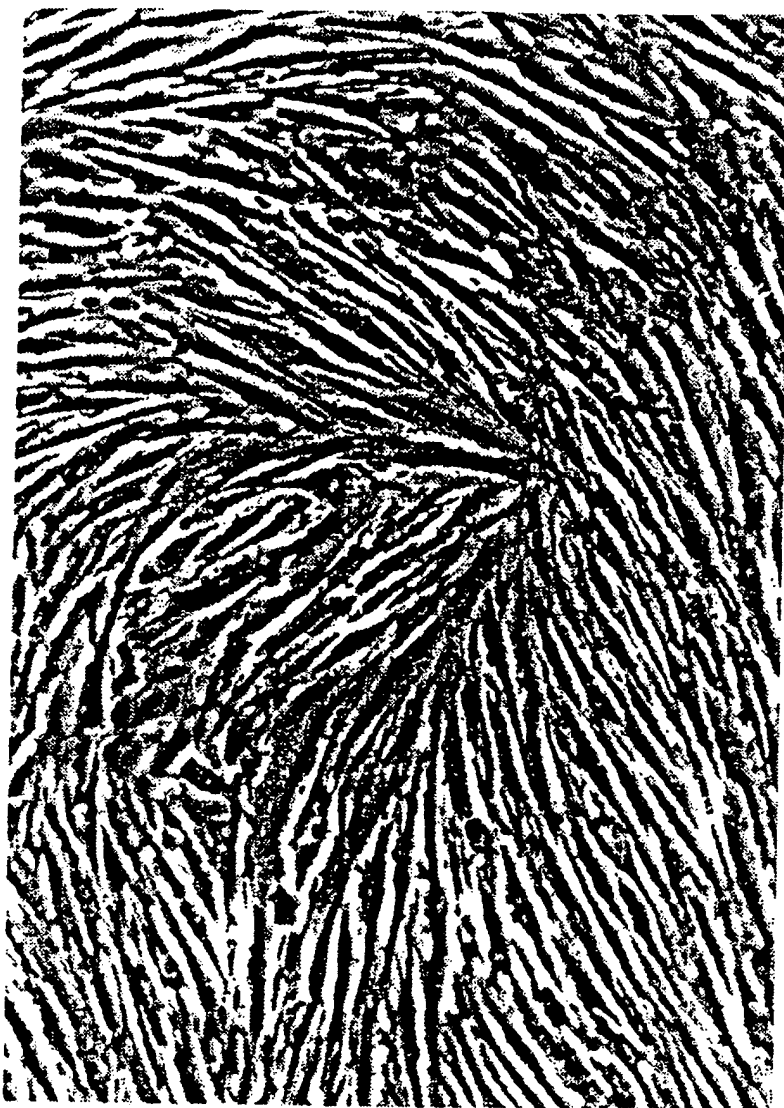


FIGURE 8

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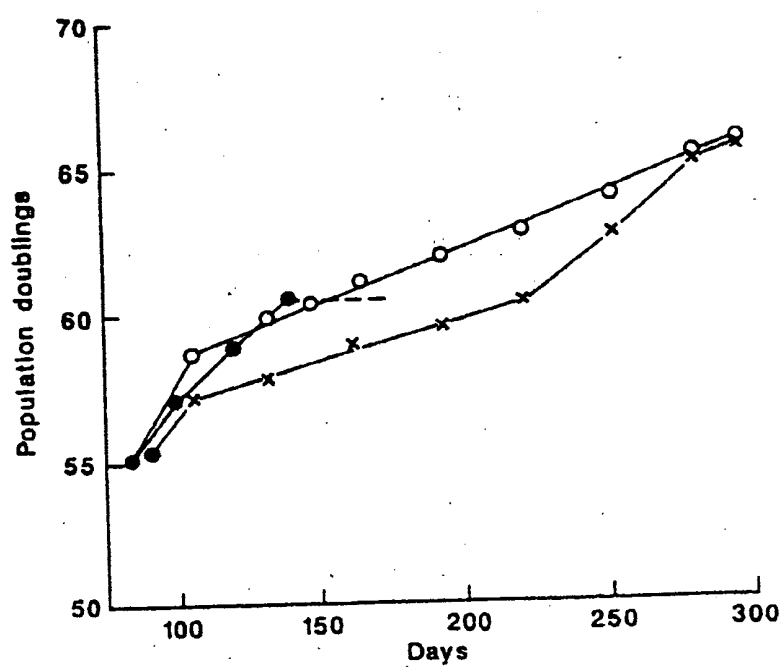


FIGURE 9

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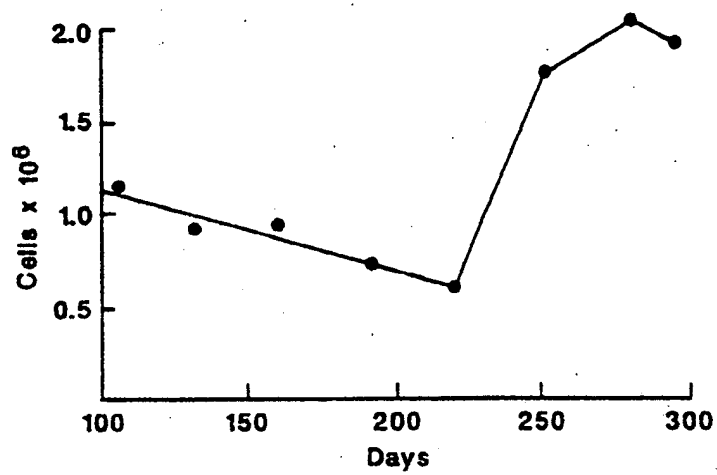


FIGURE 10

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FIGURE 11

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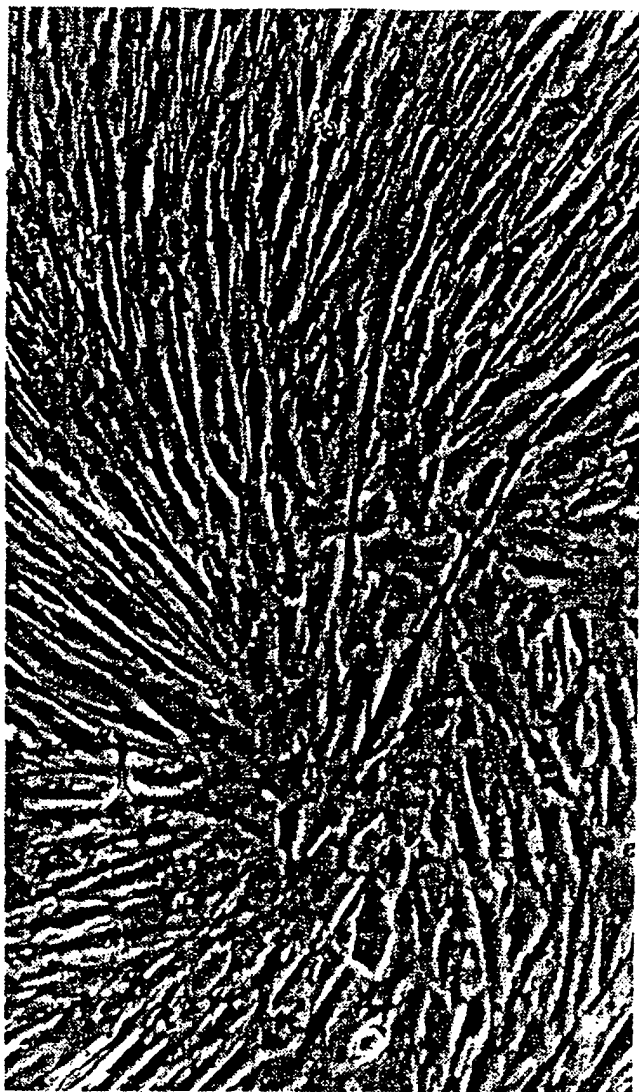


FIGURE 12

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